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Chlamydia trachomatis-Host Cell Interactions: Role of the Chlamydial Major Outer Membrane Protein as an Adhesin

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The major outer membrane protein (MOMP) of *Chlamydia trachomatis* is characterized by four symmetrically spaced variable domains (VDs I to IV) whose sequences vary among serotypes. The surface-exposed portions of these VDs contain contiguous sequences that are both serotyping determinants and in vivo target sites for neutralizing antibodies. Previous studies using surface proteolysis of *C. trachomatis* B implicated VDs II and IV of the MOMP of this serotype in the attachment of chlamydiae to host cells. In this study, we used monoclonal antibodies (MAbs) specific to antigenic determinants located in VDs II and IV of the MOMP of serotype B to further investigate the role of the MOMP in the attachment of chlamydiae to host cells. MAbs specific to serotype- and subspecies-specific epitopes located in exposed VDs II and IV, respectively, neutralized chlamydial infectivity for hamster kidney cells by blocking chlamydial attachment. We radioiodinated these MAbs and used them to determine the number and topology of the surface-exposed VDs II and IV epitopes on chlamydial elementary bodies. VDs II and IV each comprised approximately 2.86×10^4 negatively charged sites and were in proximity on the chlamydial cell surface. These studies suggest that the MAbs blocked chlamydial attachment by inhibiting electrostatic interactions with host cells. We examined the effects of thermal inactivation on both chlamydial attachment and conformation of the MOMP. Heat-inactivated chlamydiae failed to attach to host cells and exhibited a conformational change in an inaccessible invariant hydrophobic nonapeptide sequence located within VD IV of the MOMPs of *C. trachomatis* serotypes. These findings suggest that in addition to electrostatic interactions, a common hydrophobic component of the MOMP also contributes to the binding of chlamydiae to host cells. Thus, we propose that the MOMP functions as a chlamydial adhesin by promoting nonspecific (electrostatic and hydrophobic) interactions with host cells. Surface-accessible negatively charged VDs appear to be important in electrostatic binding, while the invariant region of VD IV may provide a subsurface hydrophobic depression which further promotes binding of chlamydiae to host cells through hydrophobic interactions.

Chlamydia trachomatis is an obligate intracellular gram-negative bacterium that differs from other intracellular procaryotes by its unique growth cycle. It exists as two morphologically and functionally distinct cell types: the elementary body (EB), which is infectious, and the reticulate body (RB), which is the noninfectious, vegetative form. Attachment of chlamydiae to host cells is the first step in the infectious process. Molecules that function in the attachment of the EBs to host cells have not been identified; identification of these adhesins is essential for an understanding of chlamydial pathogenesis and may aid in the development of chlamydial vaccines.

Hatch et al. (11) implicated electrostatic and hydrophobic components in the binding of chlamydiae to host cells. They suggested that the initial interaction of chlamydiae with host cells involved reversible electrostatic attractions which were enhanced by the addition of divalent cations and were followed by a more stable hydrophobic interaction. This model suggests that molecules on the surface of the EB are involved in both negatively charged electrostatic and hydrophobic interactions. One or several different molecules may contribute to these interactions.

The predominant constituents of the chlamydial outer membrane are the ca. 40-kilodalton major outer membrane protein (MOMP) (5), the 60-kilodalton and 12- to 15-kilodalton cysteine-rich proteins (10), and lipopolysaccharide (LPS) (4). The MOMP and LPS are exposed on the EB cell surface as defined by antibody binding or sensitivity to proteolytic cleavage on the surface of viable EBs. Monoclonal antibodies (MAbs) specific to the MOMP passively neutralize chlamydial infectivity for the conjunctivae of cynomolgus monkeys (8), while MAbs specific to LPS do not; this suggests that the MOMP plays an important role in chlamydial pathogenicity, perhaps as an adhesin.

The genes encoding the MOMP of *C. trachomatis* serotypes A, B, C, L1, and L2 and the *C. psittaci* meningopneumonitis (Mn), guinea pig inclusion conjunctivitis (GPIC), and enzootic abortion of ewes (EAE) strains have been sequenced (1, 16, 17, 21, 22, 31). The deduced amino acid sequences of the MOMPs of both chlamydial species are similar, with the exception of four symmetrically spaced variable domains (VDs I to IV). Epitope mapping of the *C. trachomatis* MOMPs has shown that three of these (VDs I, II, and IV) contain contiguous antigenic sites that elicit the formation of *C. trachomatis* serotyping antibodies (1, 23).

Recently, we reported that trypsin treatment of *C. trachomatis* B EBs significantly reduced their ability to attach to host cells (24). Analysis of chlamydial surface proteins showed that only the MOMP was cleaved. This cleavage was restricted to lysines 145 and 309, located in VDs II and IV, respectively. These findings led us to postulate that at the chlamydial cell surface these two domains protrude towards the external environment and may function in the attachment of chlamydiae to host cells. In this study, we used

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TABLE 1. Characteristics of antibodies specific to MOMP and LPS used in neutralization, competitive binding, and dot immunoblot assays

Antibody[a]	Location of antigenic determinant[b]	*C. trachomatis* specificity[c]	Surface accessibility as measured by:	
			Dot immunoblot assay	Susceptibility to cleavage by trypsin
PC R111	MOMP VD I; TTTGNAVAPSTLT (aa 68–80)	Subspecies	–	–
MAb B-B6	MOMP VD II; NNENQTKVSN (aa 139–148)	Serotype B	+	+
PC R183	MOMP VD II; GAFVPNMSLDQS (aa 149–160)	Subspecies	–	–
PC R2	MOMP VD III; KELPLDLTAGTDAA (aa 224–237)	Subspecies	–	–
MAb L2I-10	MOMP VD IV; TTLNPTIAG (aa 296–304)	Species	–	–
MAb B-B5	MOMP VD IV; DVKTSAE (aa 307–313)	Subspecies	+	+
MAb L2I-45	MOMP VD II; ENHATVSD (aa 143–148)	Serotype L2	+	–
MAb L2I-6	LPS	Genus	+	NA[d]
MAb EVI-H1	LPS	Genus	+	NA

[a] PC, Rabbit polyclonal antiserum.
[b] MOMP VD and amino acid (aa) sequences containing antigenic determinant.
[c] Determined by Western blot (immunoblot).
[d] NA, Not applicable.

MAbs specific to epitopes located within VDs II and IV of serotype B MOMP to define more thoroughly the role of the MOMP in chlamydia-host cell interactions.

MATERIALS AND METHODS

Chlamydiae. *C. trachomatis* serotypes B (strain B/TW-5/OT), A (strain A/Har-13), C (strain C/TW-3/OT), E (strain E/Bour), F (strain F/IC-Cal-13), G (strain F-UW-57/Cx), H (strain H/UW-4/Cx), I (strain I/UW-12/Ur), and L2 (strain LGV/L2-434) were propagated in HeLa 229 cells. EBs were purified by centrifugation in Renografin equilibrium gradients (5). Chlamydiae were metabolically radiolabeled with ^{14}C-amino acids as previously described (6).

MAbs and rabbit polyclonal antisera. MAbs were produced to the MOMP and LPS and were characterized as previously described (4, 32). Purification of MAbs from cell culture medium was done by applying clarified medium to a protein A-linked Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, N.J.) column and eluting bound immunoglobulin G with 0.25 M glycine hydrochloride buffer, pH 2.5, as described previously (4). Polyclonal antipeptide sera were prepared by immunizing rabbits with synthetic peptides conjugated to keyhole limpet hemocyanin (14). Rabbits were immunized intramuscularly with 100 μg of peptide-keyhole limpet hemocyanin conjugate in Freund complete adjuvant and boosted subcutaneously after 3 weeks with 100 μg of peptide-keyhole limpet hemocyanin conjugate in Freund incomplete adjuvant. The rabbits were bled 2 weeks after the second immunization, and their sera were assayed for antibodies to the MOMP by immunoblotting, using whole-cell lysates of all *C. trachomatis* serotypes as antigens (4). A summary of the properties of MAbs and polyclonal antisera used in this study is shown in Table 1.

Kinetic neutralization assay. Purified serotype B EBs were diluted in 0.25 M sucrose–10 mM sodium phosphate–5 mM L-glutamic acid (SPG, pH 7.2) to give a final concentration of 2.8×10^8 inclusion-forming units (IFU)/ml. Chlamydiae (0.5 ml) were mixed with MAbs serially diluted in SPG to final protein concentrations of 50, 5, and 0.5 μg/ml. The mixtures were then incubated at 37°C in a gyratory shaking water bath. Samples (20 μl) were removed from the reaction mixtures after 10, 30, and 60 min, diluted immediately with 2.0 ml of chilled SPG, and kept in an ice bath until all samples were collected. Diluted samples (200 μl) were inoculated onto 3.5×10^5 Syrian hamster kidney (HaK) cells grown on 12.5-mm glass cover slips placed in 24-well tissue culture plates. The inocula were incubated with HaK cells for 2 h at 37°C. The inocula were removed, and the monolayers were washed twice with 500 μl of Hanks balanced salt solution. Monolayers were refed with 1 ml of minimum essential medium supplemented with 10% fetal bovine serum (MEM-10) containing 0.5 μg of cycloheximide per ml and incubated at 37°C for 48 h. The monolayers were fixed with methanol and assayed for IFUs as previously described (20).

Attachment assay. To assay chlamydial attachment, a 1-ml volume of metabolically ^{14}C-amino acid-labeled EBs (6.3×10^6 IFU, 3.1×10^5 cpm/ml) was incubated with 1 ml of MAbs (100 μg of protein/ml) for 1 h at 37°C. A 200-μl volume of the mixture was inoculated onto each of four HaK cell monolayers (3.5×10^5 cells) in 24-well tissue culture plates and incubated at 4°C for 2 h. The inocula were removed, and the monolayers were washed three times with 500 μl of Hanks balanced salt solution. Two monolayer cultures were fed with MEM-10, incubated at 37°C for 48 h, and then assayed for IFUs. The remaining two monolayers were lysed with 200 μl of 0.1 N NaOH at 70°C for 1 h. The solubilized cells were collected, and the wells were washed twice with 200 μl of 0.1 N NaOH. Lysates and their corresponding washes were pooled and mixed with 10 ml of liquid scintillation cocktail (Ready-Solv; Beckman Instruments, Inc., Fullerton, Calif.), and the amount of radioactivity associated with HaK cells was determined by using a liquid scintillation counter (LS 9000; Beckman).

Quantitation of the number of MOMP molecules on the surface of chlamydiae and topology of MOMP VDs. (i) Radioiodination of MAbs. MAbs were radioiodinated by the Iodogen (Pierce Chemical Co., Rockford, Ill.) procedure as previously described (7). Briefly, a 100-μl volume of MAb (100 μg of protein) in 0.05 M sodium phosphate–0.15 M NaCl (PBS, pH 7.2) was mixed with 160 μCi of Na^{125}I in an Iodogen-coated 1-dram (~4 ml) glass vial. The reaction mixture was incubated at room temperature for 10 min with occasional mixing. Iodinated MAbs were isolated by chromatography on a PD-10 column (Sephadex G-25M; Pharmacia AB Biotechnology, Uppsala, Sweden) equilibrated, and eluted with 0.05 M sodium phosphate–0.15 M NaCl–0.5% bovine serum albumin (PBSA, pH 7.2). Specific activity (counts per minute per molecule) = (molecular weight of

immunoglobulin G/6.023 × 10^{23} molecules per mol) (counts per minute of ^{125}I/micrograms of immunoglobulin G).

(ii) Quantitation of organisms. The number of organisms was determined by acridine orange staining as described by Hobbie et al. (12). There were 5.065 × 10^6 EB particles in 5 μg of protein.

(iii) Estimation of the amount of functional radioiodinated MAbs. *C. trachomatis* B EBs (5 μg of protein, 100 μl) were incubated with ^{125}I-labeled MAbs (10^6 cpm) at 4°C for 1 h with shaking. The EBs were centrifuged at 10,000 × *g* for 10 min and washed three times with PBS, and the amounts of radioactivity in the pellet and the supernatant were determined. Serotype B EBs (5 μg of protein, 100 μl) were again added to the supernatant, reincubated, and centrifuged, and the amounts of radioactivity in the pellet and the supernatant were determined. This procedure was repeated three times until less than 10% of the total radiolabeled MAbs remaining in the supernatant became associated with the EB pellet. The percentages of functional MAb molecules and functional specific activity were estimated from the total amount of radioactivity bound to the EBs as follows: percentage of functional MAbs = (amount of radioactivity bound/total amount of radioactivity) × 100; functional specific activity = (specific activity/percentage of functional MAbs) × 100.

(iv) Determination of the number of MOMP molecules on the chlamydial surface. Serotype B EBs (5 μg of protein, 100 μl) were incubated with ^{125}I-labeled MAbs (10^6 cpm) at 4°C for 1 h with shaking. Suspensions were centrifuged and washed three times with PBS, and the amounts of radioactivity in the pellet and the supernatant were determined. The same amount of radiolabeled MAbs was added to the pellet and reincubated. This procedure was repeated four times until the EBs were saturated with MAbs. The number of MOMP molecules was calculated as follows: number of molecules of MAb bound per EB = total amount of radioactivity bound to EBs/(functional specific activity of MAb × number of EB particles). Assuming that the MAbs bound univalently, the number of MOMP molecules is equal to the number of MAbs bound; however, if some of all of the MAbs bound bivalently, the number of MOMP molecules could be up to twice this estimate. We assumed that the MAbs bound univalently and realize that this may be a low estimate of the actual number of MOMP molecules.

(v) Competitive binding assays using MAbs. Serotype B EBs (100 μl, 5 μg of protein) were mixed with unlabeled MAbs (100 μl, 100 μg of protein). The suspensions were incubated at 37°C for 30 min in a gyratory shaking water bath. A 50-μl volume of ^{125}I-labeled MAbs (10^6 cpm) was added to the appropriate antibody-EB suspension, and the mixtures were incubated at 37°C for 30 min. The suspensions were then centrifuged at 10,000 × *g* for 10 min, and the EB pellets were washed three times with PBS. The amount of ^{125}I-MAbs associated with the EBs was determined by using a Gamma 4000 (Beckman).

Incubation of *C. trachomatis* at low pH or at 56°C. (i) Infectivity assay. Serotype B EBs (2.8 × 10^9 IFU/ml) were diluted 1:10 with either SPG or 0.05 M glycine hydrochloride containing 0.15 M NaCl, pH 2.0 (glycine hydrochloride buffer). EBs suspended in SPG were heated at 56°C for 30 min or held at room temperature for 45 min. EBs suspended in glycine hydrochloride buffer were incubated at room temperature for 45 min. All samples were diluted with SPG to contain approximately 2.8 × 10^6 IFU/ml. A 200-μl volume of each suspension was inoculated onto HaK cell monolayers (3.5 × 10^5 cells) grown on glass cover slips, and the monolayers were then processed for determination of IFUs.

(ii) Attachment assay. ^{14}C-labeled *C. trachomatis* B EBs (8.2 × 10^7 IFU, 1 × 10^5 cpm/ml) were suspended in SPG and incubated at 56°C for 30 min or were incubated in 0.05 M glycine hydrochloride buffer at room temperature for 45 min. The suspensions were diluted with SPG to give a final concentration of 1.6 × 10^7 IFU/ml (2 × 10^4 cpm/ml). A 200-μl sample of each suspension was inoculated onto HaK cell monolayers which were at 4°C. The monolayers were incubated at 4°C for 2 h, the inocula were removed, the monolayers were washed three times with Hanks balanced salt solution, and the HaK cell-associated radioactivity was determined as described above.

(iii) Dot-immunoblot assay. The effect of heat or acid pH treatment on the surface antigenicity of MOMP was assessed by a previously described dot immunoblotting assay (32) using MAbs or rabbit polyclonal antisera specific to MOMP VDs I, II, III, and IV (Table 1). Briefly, organisms treated at 56°C for 30 min, incubated in pH 2.0 buffer, or incubated at room temperature were diluted with PBS to a final concentration of 5 μg of protein per ml. A 50-μl volume of these suspensions was added to wells of a BIO-DOT apparatus (Bio-Rad Laboratories, Richmond, Calif.) fitted with buffer-equilibrated nitrocellulose paper. After being blocked with 2% bovine serum albumin in PBS and washed with 0.5% Tween 20 in PBS, the nitrocellulose paper with the immobilized EBs was incubated with either MAbs or polyclonal antisera specific to MOMP. The wells were washed, and a 50-μl volume of ^{125}I-labeled protein A (5 × 10^4 cmp) (Dupont, NEN Research Products, Boston, Mass.) was added to each well. The nitrocellulose paper was washed, dried, and subjected to autoradiography to detect antibodies bound to the EB cell surface.

(iv) Incubation of representative *C. trachomatis* serotypes at 56°C. *C. trachomatis* serotypes A, C, E, F, G, H, I, and L2 were diluted 1:10 with SPG and incubated at 56°C for 30 min. After incubation, the suspensions were diluted with SPG to contain approximately 1.8 × 10^6 to 8.0 × 10^6 IFU/ml. Infectivity was assayed by using HaK cell monolayers, and immunoreactivity with MAb L2I-10 was assayed by using dot immunoblotting as described above.

Inhibition of chlamydial infectivity by synthetic peptides. Peptides were synthesized by solid-phase techniques by using the R^{a}MPS multiple peptide synthesis system (E.I. du Pont de Nemours and Company, Wilmington, Del.) as directed by the manufacturer. Peptides corresponding to amino acid residues 139 to 154 (VD II), 296 to 304 (VD IV), and 304 to 312 (VD IV) of the *C. trachomatis* serotype B MOMP were synthesized and used in inhibition experiments. These peptides contain the epitopes recognized by MAbs B-B6, L2I-10, and B-B5, respectively. Synthetic peptides were diluted with SPG to 1,000, 100, 10, and 1 μM concentrations. Purified *C. trachomatis* B EBs were diluted in SPG to give a final concentration of 2.8 × 10^6 IFU/ml. Suspensions of EBs were mixed with either peptides, MAb B-B5 (100 μg/ml), or SPG. Aliquots (200 μl) of the suspensions were inoculated onto HaK cell monolayers (3.5 × 10^5 cells) and incubated at 4°C for 2 h. The monolayers were then washed, fed with MEM-10, and processed for determination of IFUs as described above.

RESULTS

Neutralization of infectivity of chlamydiae for HaK cells. MAbs B-B5 and B-B6, which react with surface-accessible MOMP epitopes on viable *C. trachomatis* B EBs, and MAb L2I-10, which reacts with an unaccessible MOMP epitope

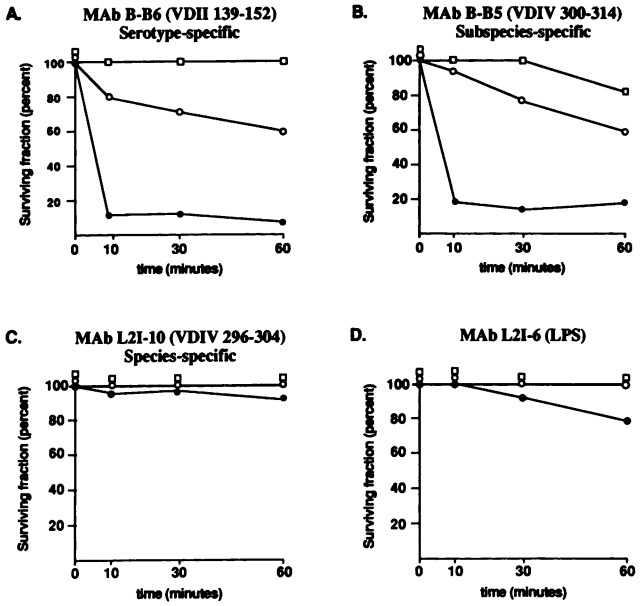

FIG. 1. Neutralization of *C. trachomatis* serotype B infectivity for HaK cells with anti-B-MOMP and anti-LPS MAbs. Each MAb was diluted with SPG to yield final MAb concentrations of 100 (●), 10 (○), and 1 μg/ml (□) and mixed with an equal volume of serotype B EBs (2.8×10^8 IFU/ml). Controls were EBs incubated with SPG. Samples (20 μl) were removed at the indicated times, diluted with SPG, and assayed for residual infectivity. A mixture of EBs and SPG buffer was used to determine the zero time values.

(Table 1), were examined for their abilities to neutralize infectivity for HaK cells. Each MAb was diluted to give a final protein concentration of 50, 5, or 0.5 μg/ml and examined in a kinetic neutralization assay. MAbs B-B6 and B-B5, specific to epitopes located in MOMP VDs II and IV, respectively, neutralized infectivity, whereas MAb L2I-10, specific to an epitope located in VD IV (not immunoaccessible on the EB surface), and the LPS-specific MAb L2I-6 did not (Fig. 1). MAbs B-B6 and B-B5 exhibited similar rates of inactivation and neutralized infectivity to nearly the same extent. At 50 μg/ml, EB infectivity was reduced by 80% or greater, with maximal neutralization occurring after 10 min of incubation of antibody with EBs. At 5 μg/ml, both MAbs exhibited much slower rates of inactivation and resulted in a larger fraction of unneutralized EBs. Minimal to no neutralization was observed with MAbs B-B6 and B-B5 at concentrations of 0.5 μg/ml.

Inhibition of chlamydial attachment as a mechanism of neutralization. Antibody binding to the MOMP may inhibit chlamydial infectivity by several mechanisms, including inhibition of attachment, penetration, or differentiation of EBs to RBs. To differentiate among these possible mechanisms, we tested the ability of anti-MOMP MAbs to prevent chlamydial attachment. Intrinsically ^{14}C-radiolabeled EBs were mixed with MAbs B-B6, B-B5, or L2I-6 at a final concentration of 50 μg of protein per ml and incubated at 37°C for 60 min. Suspensions of MAbs and chlamydiae were inoculated onto monolayers of HaK cells, incubated, and washed, and attachment was determined by measuring the radioactivity associated with HaK cells. MAbs B-B6 and B-B5 inhibited the binding of EBs to HaK cells by 85 and 65%, respectively (Table 2). MAb L2I-6 did not inhibit the binding of EBs to HaK cells; therefore, MAbs reactive with surface-exposed MOMP epitopes located in VDs II and IV neutralized infectivity by preventing chlamydial attachment. Reduction of attachment was not due to aggregation in the presence of MAbs, as MAb L2I-6, which binds to the chlamydial surface, did not block attachment. Thus, binding of MAbs to the chlamydial surface under these conditions did not promote agglutination.

Estimation of the number of MOMP molecules and the topologies of VDs II and IV. The percentage of functional radioiodinated MAbs was determined by repeated incubation of MAbs with EBs until less than 10% of the MAbs could be absorbed with fresh EBs. The amount of functional antibody molecules was determined from the amount of radioactivity associated with the EB pellet. Of the MAbs in each MAb preparation, 15 to 21% were functional (Table 3). The number of MOMP molecules at the chlamydial surface was determined by saturating the EBs with successive incubation with MAbs. After the fourth incubation with fresh MAbs, the EB surface was saturated with MAbs. From the amount of radioactivity associated with the EBs, we estimate that the number of MOMP epitopes for each MAb on each EB particle was approximately 2.86×10^4 molecules (Table 4). This number corresponded to approximately 10^5 molecules per μm^2 of EB surface area and is comparable with the number of *Escherichia coli* porin proteins on the surfaces of these cells (4.8×10^4 molecules per um^2) (13). We investigated the topologies of VDs II and IV at the EB surface by using a MAb competitive binding assay. Serotype B EBs were incubated with unlabeled MAbs (B-B6, B-B5, or EVI-H1) and then incubated with radioiodinated MAbs. The amount of radiolabel associated with EBs was determined, and the percent inhibition of binding was calculated (Table 5). Homologous inhibition of binding to EBs was observed for all MAbs. MAb B-B6 effectively inhibited the binding of MAb B-B5, and conversely, MAb B-B5 inhibited the binding of MAb B-B6. MAb EVI-H1 (LPS specific) did not inhibit the binding of the MOMP-specific MAbs. The abilities of MAbs B-B6 and B-B5 to inhibit the binding of each other indicated that VDs II and IV are in close proximity on the EB surface.

The effect of heat treatment on MOMP conformation and chlamydial attachment. Chlamydial infectivity is readily destroyed upon exposure of EBs to environmental changes

TABLE 2. Inhibition of chlamydial attachment to HaK cells with MAbs

MAb	Amt of ^{14}C-EBs inoculated (cpm)[a]	Amt of ^{14}C-EBs bound (cpm [% of total cpm of ^{14}C-EBs added])[a]	% Reduction
Control (SPG buffer)	$3.2 \times 10^4 \pm 1.2 \times 10^3$	$7.4 \times 10^3 \pm 6.5 \times 10^2$ (23)	
B-B6	$3.1 \times 10^4 \pm 3.5 \times 10^2$	$1.1 \times 10^3 \pm 1.2 \times 10^2$ (3.4)	85
B-B5	$3.0 \times 10^4 \pm 1.0 \times 10^3$	$2.5 \times 10^3 \pm 4.8 \times 10^2$ (8.1)	65
L2I-6	$3.3 \times 10^4 \pm 2.9 \times 10^3$	$9.0 \times 10^3 \pm 1.2 \times 10^3$ (27)	0

[a] Results are means ± standard errors of triplicate samples.

TABLE 3. Estimated functional activities of radioiodinated MAbs

MAb	Serotype	Amt of ^{125}I-MAb added (cpm)[a]	Amt of ^{125}I-MAb bound to EBs (cpm) after incubation no.:			Total amt of ^{125}I-MAb bound (cpm)[b]	% Functional activity
			1	2	3		
B-By	B	$6.7 \times 10^5 \pm 4.9 \times 10^4$	1.2×10^5	1.3×10^4	6.4×10^3	1.4×10^5	21
B-B5	B	$7.8 \times 10^5 \pm 6.2 \times 10^4$	1.5×10^5	6.5×10^3	2.1×10^3	1.6×10^5	20
EVI-H1	B	$9.9 \times 10^5 \pm 6.6 \times 10^3$	1.4×10^5	3.4×10^4	1.56×10^4	1.9×10^5	20
L2I-45[c]	B	$7.7 \times 10^5 \pm 3.3 \times 10^4$	5.3×10^3	4.4×10^3	3.9×10^3	1.3×10^4	
L2I-45	L2	$7.7 \times 10^5 \pm 3.3 \times 10^4$	5.15×10^5	4.0×10^4	2.4×10^4	5.6×10^5	15

[a] Results are means ± standard errors of duplicate samples.
[b] Represents the total amount of functional antibody molecules.
[c] MAb L2I-45 does not bind to serotype B EBs. This MAb was used to determine the amount of nonspecific binding in this assay and was less than 2%.

(pH and heat treatment) (3; unpublished observations). We therefore determined whether physical treatments of EBs that result in a loss of infectivity could be associated with conformational changes in the MOMP at the EB cell surface. EBs were heated at 56°C for 30 min or exposed to pH 2.0, and the effects of these treatments on chlamydial attachment and MOMP conformation, as assessed by changes in surface antigenicity, were determined (Fig. 2). Heated and acid-treated EBs were probed with antibodies that bind to contiguous antigenic determinants located in each of the MOMP VDs. Since these domains are symmetrically located in the primary sequence of the MOMP, changes in their reactivities with antibodies provide a reasonable representation of conformational changes that may occur in the total protein. All antibodies showed identical dot immunoblot reactivities with control, heated, and pH 2.0-treated EBs except MAb L2I-10 (Fig. 2), which binds to the species-specific nonapeptide TTLNPTIAG (amino acids 296 to 304) located in the central region of VD IV of all serotypes except K, where threonine replaces alanine at residue 303, a conservative change. This nonapeptide epitope, which is inaccessible to antibody on untreated, viable EBs, became immunoaccessible at the cell surfaces of heated EBs. The striking difference in the surface exposure of the conserved species-specific epitope after heat treatment suggested that a conformational change had occurred in VD IV. The immunoreactivity of antibodies specific to determinants located in VDs I, II, III, and the C-terminal end of VD IV were not changed by heating, indicating that the conformational changes which occurred in MOMP were localized to the conserved nonapeptide region of VD IV. Exposure of EBs to pH 2.0 resulted in a very weak reactivity with MAb L2I-10 but did not cause the striking conformational change in VD IV that was observed in heat-inactivated EBs.

The infectivity of EBs was reduced by 100% after treatment at pH 2.0 or heating at 56°C for 30 min (Table 6). However, significant differences were found in the abilities of EBs inactivated by these two treatments to attach to HaK cells. Heat-treated, intrinsically radiolabeled EBs showed an 81% reduction in their ability to bind to HaK cells compared with control EBs. EBs inactivated by pH 2.0 treatment showed a 28% reduction in attachment compared with control organisms. This slight decrease in attachment is consistent with the moderate change in surface exposure of the species-specific determinant observed by dot immunoblot for pH 2.0-inactivated EBs (Fig. 2).

We used serotype B EBs for these studies because of the availability of an extensive panel of MAbs and polyclonal monospecific antisera specific to its MOMP. These findings, however, were not unique to this serotype. We examined the effect of heating on both the infectivity of EBs and the immunoreactivity of MAb L2I-10 on eight representative *C. trachomatis* serotypes and found them identical to that reported here for serotype B. Heating EBs of each serotype at 56°C for 30 min destroyed their infectivities for HaK cells and resulted in surface exposure of the normally inaccessible species-specific nonapeptide epitope at their cell surfaces (Fig. 3).

Inhibition of chlamydial infectivity by synthetic peptides. To determine whether the contiguous amino acid sequences of VDs II and IV were directly involved in the chlamydial attachment to host cells, we attempted to inhibit chlamydial cell attachment by using synthetic peptides corresponding to the VD sequences in which the MOMP-neutralizing sites were located. Peptides were mixed with EBs and then inoculated onto cells. Peptides did not inhibit the binding and infectivity of chlamydiae (data not shown). Thus, attachment of chlamydiae to host cells involves a more complex process than the direct binding of short contiguous oligopeptide regions of the MOMP. The binding of the MOMP to host cell receptors may be mediated by electrostatic interactions contributed by the charged hydrophilic residues contained within the VDs and by hydrophobic interactions with the species-specific nonapeptide sequence. These interactions may depend upon the presence of a repetitive mosaic of MOMP epitopes on the surface of the EB.

TABLE 4. Quantitation of MOMP and LPS molecules on the chlamydial surface

MAb	Functional sp act (cpm/molecule)	No. of EBs/ incubation	Amt of ^{125}I-MAb bound (cpm)	Molecules of ^{125}I-MAb bound per EB: molecules of MOMP or LPS on EB
B-B6	1.9×10^{-6}	5.065×10^6	2.85×10^5	2.91×10^4
B-B5	1.75×10^{-6}	5.065×10^6	2.48×10^5	2.80×10^4
EVI-H1	1.75×10^{-6}	5.065×10^6	3.00×10^5	3.38×10^4

DISCUSSION

Our previous studies demonstrated that tryptic cleavage within VDs II and IV of serotype B MOMP prevented chlamydial attachment (24), suggesting that these exposed domains may be critical for chlamydial infectivity and that these structures, either separately or cooperatively, function in the attachment of chlamydiae to host cells. Here we provide further evidence supporting the function of MOMP VDs in chlamydial attachment. MAbs B-B6 and B-B5, which are specific for serotype- and subspecies-specific epitopes located in MOMP VDs II and IV of serotype B, respectively, neutralized infectivity of EBs for HaK cells by preventing

TABLE 5. Competitive binding of MAbs specific to MOMP surface-accessible epitopes[a]

Inhibiting MAb (unlabeled)	Results with ^{125}I-labeled MAb					
	B-B6		B-B5		EVI-H1	
	cpm bound[b]	% Inhibition	cpm bound[b]	% Inhibition	cpm bound[b]	% Inhibition
B-B6 (B-MOMP VD II)	$5.4 \times 10^3 \pm 5.5 \times 10^2$	95	$1.16 \times 10^4 \pm 4.0 \times 10^3$	89	$9.7 \times 10^4 \pm 2.0 \times 10^3$	1.5
B-B5 (B-MOMP VD IV)	$1.2 \times 10^4 \pm 1.3 \times 10^3$	88	$6.8 \times 10^3 \pm 2.5 \times 10^2$	94	$1.04 \times 10^5 \pm 1.2 \times 10^4$	0
EVI-H1 (LPS)	$1.1 \times 10^5 \pm 8.5 \times 10^3$	0	$1.3 \times 10^5 \pm 1.1 \times 10^4$	0	$7.07 \times 10^3 \pm 6.8 \times 10^2$	93
L2I-45 (L2-MOMP VD II)	$1.05 \times 10^5 \pm 3.5 \times 10^3$		$1.06 \times 10^5 \pm 8.7 \times 10^3$		$9.8 \times 10^4 \pm 6.8 \times 10^3$	

[a] Unlabeled MAb (100 μl; 100 μg of protein) was mixed with 100 μl of serotype B EBs (5 μg of protein) at 37°C for 30 min; ^{125}I-labeled MAbs (10^6 cpm) were added to the mixture of unlabeled MAbs and serotype B EBs and incubated at 37°C for 30 min. The suspensions were then centrifuged, and the pellets were washed three times with PBS. The amount of ^{125}I-MAbs associated with the EBs was determined. The amount of ^{125}I-MAb bound when MAb L2I-45 was used as the inhibitory MAb was considered to be the 100% binding value.

[b] Results are means ± standard errors for duplicate samples. The values shown are from a single experiment; this experiment has been performed three times with identical results.

chlamydial attachment. VDs II and IV, though distal to one another in the MOMP primary sequence, are in proximity on the chlamydial cell surface (Table 5), and each comprises approximately 2.86×10^4 antibody-binding sites. We showed that the hydrophobic nonapeptide TTLNPTIAG (amino acids 296 to 304) region central to VD IV, which is conserved among the MOMPs of different *C. trachomatis* serotypes, maintains a specific conformation near the EB cell surface. The conformation of this domain is altered upon heating EBs, as shown by a change in the immunoaccessibility of the invariant nonapeptide sequence, and this change was associated with a dramatic reduction in the ability of EBs to attach to host cells. Taken together, these findings strongly support a role for the MOMP in chlamydial attachment to host cells; however, the precise mechanism(s) by which the MOMP mediates attachment is unclear.

We propose that the MOMP functions in the attachment of chlamydiae to host cells by the following two independent and perhaps cooperative mechanisms: (i) electrostatic interactions which involve surface-exposed VDs and (ii) hydrophobic interactions of the inaccessible, hydrophobic, invariant nonapeptide sequence located in VD IV. Electrostatic and hydrophobic interactions may be important in the attachment of chlamydiae (11). The chlamydial cell surface is negatively charged (with a pI of 4.25 to 5) (15, 25), and attachment of both *C. trachomatis* and *C. psittaci* strains to eucaryotic cells is significantly increased by the divalent cations Ca^{2+} and Mg^{2+} (11). These molecules may reduce electrostatic repulsion, perhaps by acting as a bridge to allow cellular contact between chlamydiae and host cells.

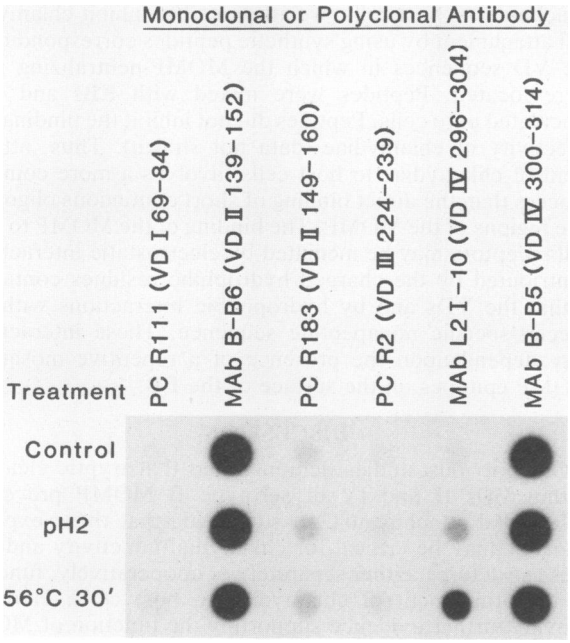

FIG. 2. Dot immunoblots of serotype B EBs treated at pH 2.0 or 56°C for 30 min (30'). Control or treated EBs were probed with MAbs or polyclonal (PC) anti-peptide sera specific to accessible or inaccessible determinants in MOMP VDs I, II, III, and IV.

Electrostatic binding requires a high density of negatively charged residues on the surface of the EB. VD II and IV sequences, which have a net negative charge, occur with a density of 10^5 epitopes per μm^2 of EB surface area, thus providing a negative charge to the EB. Antibodies binding to these domains may block chlamydial attachment directly by preventing these charge-dependent interactions. Synthetic peptides corresponding to sequences within these variable domains do not inhibit attachment, since peptides in solution would not form the high density of negative charges required for this type of electrostatic interaction. The differences in primary amino acid sequences of the MOMP VDs of different chlamydial serotypes representing serotype-specific sites for neutralizing antibodies may seem paradoxical with regard to a common function. However, despite such sequence variation, each surface-exposed domain in all serotypes maintains a negative charge and hydrophilic properties (30). VDs I and II have different surface exposures, which appear to be a property of the serotypes within a given serogroup. For serotypes A, C, and I within the C serogroup, VDs I but not II are surface accessible and are the location of the divergent sequences that are major sites for neutralizing antibodies (1; N.G.W., unpublished observations). In contrast, VDs II but not I of the MOMPs of B-complex serotypes B, Ba, and L2 are surface accessible and are the primary serotype-specific sites for neutralizing antibodies for these serotypes. Although the exposure and antigenic properties of these domains in the MOMPs of other serotypes has yet to be defined, the amino acid sequences of their VDs have been determined (30). Examination of the amino acid compositions showed that the VDs I of the MOMPs of serotypes within the C serogroup are each negatively charged, while the VDs II of these serotypes are either uncharged or positively charged. Similarly, VDs II of serotypes within the B serogroup are negatively charged, while VDs I are either uncharged or positively charged.

TABLE 6. Effect of heating and pH 2.0 treatment on the infectivity and binding properties of serotype B EBs for HaK cells[a]

Treatment	Infectivity (IFUs/3.5 × 10^5 HaK cells[b] [% reduction])	Amt inoculated (cpm) onto HaK cells[b]	Amt (%) bound (cpm) to HaK cells[b]	% Reduction
None	$2.8 \times 10^4 \pm 1.5 \times 10^3$	$4.7 \times 10^3 \pm 2.2 \times 10^2$	$8.45 \times 10^2 \pm 2.1 \times 10^1$ (18)	
pH 2.0	0 (100)	$4.35 \times 10^3 \pm 9.8 \times 10^1$	$5.72 \times 10^2 \pm 5.0 \times 10^1$ (13)	28
56°C for 30 min	0 (100)	$5.2 \times 10^3 \pm 3.4 \times 10^1$	$1.82 \times 10^2 \pm 9.0 \times 10^1$ (3.5)	81

[a] ^{14}C-labeled EBs were inoculated onto HaK cell monolayers, and the amount of radioactivity bound was determined as described in Materials and Methods.
[b] Results are means ± standard errors of triplicate samples.

Exposed regions of VDs IV (C-terminal end), which are accessible on all serotypes (33), are also negatively charged (30). Therefore, the model we propose here, that negatively charged divergent sequences in exposed MOMP VDs function in the binding of chlamydiae to host cells via electrostatic interactions, is consistent with the known surface exposure and charge properties of the MOMP VDs for all *C. trachomatis* serotypes. Thus, although sequence variations have occurred in these regions, producing different antigenic determinants which are serotyping and neutralizing epitopes, each site has conserved a net negative charge, a finding which supports a common and critical function for these domains in chlamydial pathogenicity.

We hypothesize that electrostatic binding is followed by a more specific hydrophobic interaction involving the invariant nonapeptide sequence located in VD IV. We showed that mild heat treatment of EBs significantly reduces their ability to attach to cells. This functional change was correlated with a conformational reorganization in VD IV that involved the invariant species-specific nonapeptide (TTLNPTIAG [amino acid 296 to 304]). This nonapeptide, which is normally inaccessible to antibody on native EBs, became immunoaccessible on heat-treated organisms. These correlative findings provide indirect evidence that this invariant region of VD IV functions as a cryptic hydrophobic chlamydial binding site. There are several properties of VD IV and its central nonapeptide portion that implicate it as a potential binding site in chlamydial attachment. VD IV is surface exposed on viable EBs of all *C. trachomatis* serotypes, implying that it may have an important function in chlamydia-host cell interactions. The hydrophobic nonapeptide species-specific epitope (TTLNPTIAG [amino acids 296 to 304]) located in the central region of VD IV is highly conserved among both *C. trachomatis* and *C. psittaci* strains (Fig. 4). Among *C. trachomatis* serotypes, the only variation within this nonapeptide sequence is the substitution of threonine for alanine at residue 303 in the MOMP of serotype K. The *C. psittaci* strains for which the MOMP sequences are known contain several conservative substitutions within this nonapeptide region (tryptophan for leucine, leucine for isoleucine, and serine for threonine). The only substitution which does not conserve chemical properties is the substitution of either leucine or isoleucine for alanine at residue 303. This hydrophobic nonapeptide is flanked at both its N- and C-terminal ends by hypervariable domains containing hydrophilic and charged residues, suggesting that the invariant sequence may form a hydrophobic depression or cleft. On the basis of these properties, this invariant region of VD IV may be a

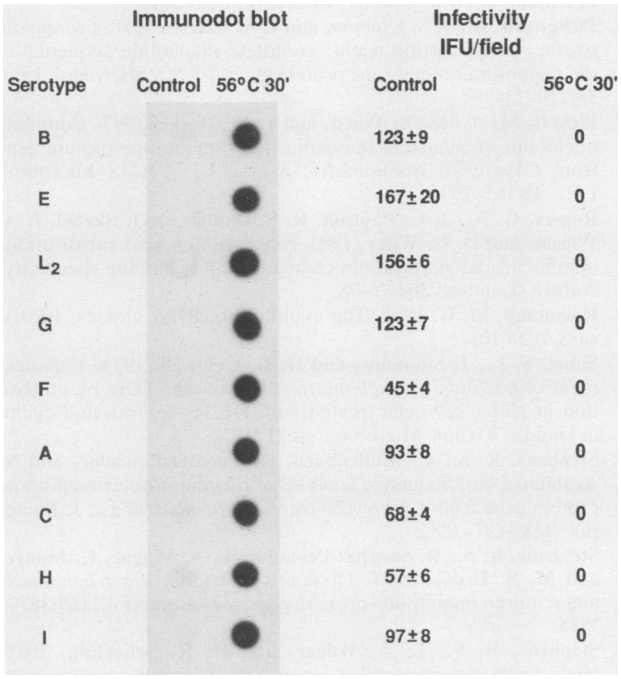

FIG. 3. *C. trachomatis* serotype B, E, and L2 (B group), G and F (intermediate group), and A, C, H, and I (C group) EBs were treated at 56°C for 30 min (30') and tested for immunoreactivity to MAb L2I-10 (dot blot) and infectivity for HaK cells.

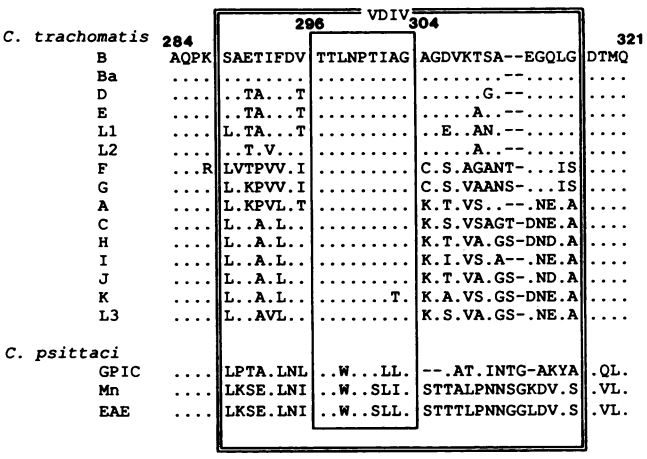

FIG. 4. Amino acid sequences of VD IV of the MOMPs of all 15 *C. trachomatis* serotypes and three *C. psittaci* strains (16, 30, 31). The central boxed region is the nonapeptide sequence containing the species-specific epitope of *C. trachomatis* serotypes recognized by MAb L2I-10. The hydrophobic nonapeptide sequence is strictly conserved among all *C. trachomatis* serotypes except serotype K, which has a T-for-A substitution at residue 303. The nonapeptide sequence in the three *C. psittaci* strains contains several amino acid substitutions; however, each is a hydrophobic residue, thereby maintaining the hydrophobic properties of the nonapeptide sequence between the MOMPs of the two chlamydial species.

cryptic binding site that functions in the attachment of chlamydiae to host cells. In this model, all chlamydiae utilize a common binding domain that is protected from immune surveillance. The protruding sequences of VD IV flanking this region would be expected to exhibit sequence variation, since they are surface-exposed, charged sites and therefore primary targets for immunological attack.

The mechanisms we propose for the MOMP in functioning as a chlamydial adhesin involve nonspecific factors. These are clearly important virulence determinants, since they provide a mechanism for the initial interactions of chlamydiae with host cells. However, adhesins which interact with host cell ligands through molecular complementarity are likely required to initiate specific binding of chlamydiae to host cells. One possibility is that the amino acid residues within the hydrophobic region of VD IV confer receptor specificity for binding. We have no evidence to support this hypothesis; however, amino acid substitutions occur in the nonapeptide region between *C. trachomatis* and *C. psittaci* strains, which could provide differences in host cell receptor specificities that would explain the differences in host tropism exhibited by members of the two chlamydial species. This hypothesis is similar to the mechanisms proposed for the binding of the hemagglutinin molecule of influenza virus (18, 26, 28, 29) and the capsid proteins of picornavirus (19) to eucaryotic cells. The influenza virus hemagglutinins attach to the sialic acid residues of host cell membrane receptors. The sialic acid-binding site on the hemagglutinin molecule is located in a pocket on the distal tip of the molecule. Indeed, it would not be surprising if chlamydiae, being obligate intracellular organisms, have developed strategies similar to those of viruses for infecting eucaryotic cells. Alternatively, the attachment of chlamydiae may involve multiple adhesins, and specific binding to host cell receptors may be a function of surface molecules other than the MOMP. Hackstadt (9) and Wenman and Meuser (27) have identified 18- and 32-kilodalton chlamydial proteins that bind to radiolabeled HeLa cell membrane components. It is possible that these proteins function in the specific binding of chlamydiae to host cells through receptor-ligand interactions.

Beachey (2) has provided criteria for proving that a specific bacterial molecule is an adhesin mediating bacterial binding to cells. Indirect evidence can be demonstrated through inhibition of adherence by (i) adhesin analogs, (ii) enzymes or chemicals that destroy the adhesin, and (iii) antibodies specific for the adhesin. This study and our previous study showing that tryptic cleavage of MOMP at the chlamydial surface prevents chlamydial attachment (24) provide strong indirect evidence for the MOMP as a chlamydial adhesin.

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